Deer as a potential wildlife reservoir for Parachlamydia species

Regenscheit, Nadine ; Holzwarth, Nathalie ; Greub, Gilbert ; Aeby, Sébastien ; Pospischil, Andreas ; Borel, Nicole

Abstract: Wildlife populations represent an important reservoir for emerging pathogens and transboundary livestock diseases. However, detailed information relating to the occurrence of endemic pathogens such as those of the order Chlamydiales in such populations is lacking. During the hunting season of 2008, 863 samples (including blood, conjunctival swabs, internal organs and faeces) were collected in the Eastern Swiss Alps from 99 free-living red deer (Cervus elaphus) and 64 free-living roe deer (Capreolus capreolus) and tested using ELISA, PCR and immunohistochemistry for members of the family Chlamydiaceae and the genus Parachlamydia. Parachlamydia spp. were detected in the conjunctival swabs, faeces and internal organs of both species of deer (2.4% positive, with a further 29.5% inconclusive). The very low occurrence of Chlamydiaceae (2.5%) was in line with serological data (0.7% seroprevalence for Chlamydia abortus). Further investigations are required to elucidate the zoonotic potential, pathogenicity, and distribution of Parachlamydia spp. in wild ruminants.

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Institut für Veterinärpathologie
der Vetsuisse-Fakultät, Universität Zürich

Direktor: Prof. Andreas Pospischil

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Nadine Regenscheit

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Short Communication

Potential Wildlife Reservoir for *Parachlamydia* in Red Deer (*Cervus elaphus*) and Roe Deer (*Capreolus c. capreolus*)

Nadine Regenscheit [a], Nathalie Holzwarth [a], Gilbert Greub [b], Sebastien Aeby [b], Andreas Pospischil [a], Nicole Borel [a,*]

[a] *Institute of Veterinary Pathology, University of Zurich, Vetsuisse Faculty Zurich, Winterthurerstrasse 268, CH-8057 Zurich, Switzerland*
[b] *Center for Research on Intracellular Bacteria, Institute of Microbiology, University Hospital Center and University of Lausanne, Rue du Bugnon 21, CH-1011 Lausanne, Switzerland*

* Corresponding author. Tel.: +41 44 6358576
Email address: n.borel@access.uzh.ch (N. Borel).

Abstract

Wildlife populations represent an important reservoir for emerging pathogens and trans-boundary livestock diseases. However, exact knowledge on common domestic pathogens such as *Chlamydiales* in these populations is lacking. During the hunting season of 2008, 863 samples, including blood, eye swabs, organs and fecal samples, from 99 red deer and 64 roe deer were collected in the eastern Swiss Alps, and samples were tested by ELISA, PCR and immunohistochemistry for *Chlamydiaceae* and *Parachlamydia*. *Parachlamydia* was detected in eye swabs, fecal samples and organs of free-living deer (2.4% positives and 29.5% questionable positives). The very low occurrence of *Chlamydiaceae* (2.5%) was in agreement with the few positive serological results (0.7% seroprevalence for *C. abortus*). Further investigations on *Parachlamydia* are needed to elucidate the zoonotic potential, the pathogenicity, and distribution in wild ruminants.

Keywords: Chlamydiaceae; Parachlamydia; Red deer; Roe deer; Zoonotic Potential

Ovine enzootic abortion (OEA) caused by *Chlamydia (C.) abortus* is a worldwide and major cause of abortion in small ruminants. *C. abortus* is the most common infectious abortigenic agent in sheep and goats in Switzerland (Chanton-Greutmann et al., 2002). As domestic and wild ruminants are grazing on the same alpine pastures, interactions are likely to occur, favoring transmission of infectious agents (Ryser-Degiorgis et al., 2002).

Chlamydia-like organisms as pathogens of humans and animals have been described including *Parachlamydia (P.) acanthamoebae* (Borel et al., 2010), and abortions in ruminants due to *P. acanthamoebae* have been recently reported in Switzerland and Scotland (Borel et al., 2010; Deuchande et al., 2010). However, data for *Parachlamydia* in wild ruminants are lacking so far. Therefore, the red deer (*Cervus elaphus)* and the roe deer (*Capreolus c. capreolus*) were tested in this study to elucidate the occurrence of *Chlamydia* and *Parachlamydia* in cervids.

During the hunting season of 2008 (September to December), 4'384 red deer and 3'274 roe deer were hunted (Annual report hunting, 2008). All investigated samples were collected in the geographical region Surselva (including side valleys) of the canton of Grisons in the eastern part of the Swiss Alps (46°36 to 46°4 N, 8°42 to 9°20 E). For each animal, the signalement (species, sex, age), body condition and the coordinates of sampling were recorded by the gamekeepers and hunters. The samples originated from the two free-living species of the family *Cervidae* in Switzerland *(n=163)*: Roe deer (*Capreolus c. capreolus*) *(n=64)* and red deer (*Cervus elaphus*) *(n=99)*. In total, 863 samples out of 163 hunted deer were collected including sera *(n= 146)*, EDTA blood *(n=147)*, eye swabs from both eyes *(n=306)*, organ samples (liver, lung) *(n=204)* and fecal samples *(n=60)*.

DNA extraction of eye swabs *(n=306)* was performed as previously described (Holzwarth et al., 2011). The pretreatment of the EDTA blood *(n=147)* consisted of a one to four dilution with Hanks' Balanced Salt Solution (HBBS) (Invitrogen AG). 200 µL of each pretreated eye swab and EDTA blood sample was used for automatic DNA extraction by the MagNA Pure LC System (Roche Diagnostics). Extraction of the organ *(n=204)* and fecal samples *(n=60)* was performed using a commercially available DNeasy Blood and Tissue Kit (QIAGEN). All samples *(n=717)* were examined on an ABI 7500 instrument (Applied Biosystems) applying the 23S rRNA-based *Chlamydiaceae* family-specific real-time PCR as described previously (Ehricht et al., 2006). This method includes primers Ch23S-F (5'-CTGAAACCAGTAGCTTATAAG CGGT-3'), Ch23S-R (5'-ACCTCGCCGTTTAACTTAACTCC-3'), and probe Ch23S-p (FAM-CTCATCA TGCAAAAGGCACGCCG-TAMRA) and an internal amplification control consisting of primers EGFP-1-F (5'-GACCACTACCAGCAGAACAC-3'), EGFP-10-R (3'-CTTGTACAGCTCGTCCATGC-5') and probe EGFP-HEX (HEX-AGCACCCAGTCCGCCCTGAGCA-BHQ1). A 111-bp product specific for members of the family *Chlamydiaceae* is produced as well as a 177-bp product for the internal amplification control. If inhibition of the internal control was observed, the sample was retested at a dilution of 1:10.

A cycle threshold (Ct value) of ≤ 38.0 was considered as positive. All positive and questionable samples were further investigated by ArrayTube Microarray (AT) (Alere) as described previously (Borel et al., 2008). Samples positive by real-time PCR for *Chlamydiaceae* but negative by AT were investigated by a PCR method amplifying a 298-bp product of the 16S rRNA gene specific for the order *Chlamydiales* (Everett et al., 1999). The

obtained sequences were compared with those available in GenBank by using the BLAST server from the National Center for Biotechnology Information[1].

The possible presence of *Parachlamydia sp.* in eye swabs *(n=306)*, fecal samples *(n=60)* and organ samples *(n=204)* was examined by a recently described real-time PCR assay (Casson et al., 2008) with minor modifications. A Ct value of ≤ 35.0 was considered as positive and Ct values between 35.0 and 38.0 were considered as questionable positive. Formalin-fixed and paraffin-embedded lung and liver from real-time PCR positive or questionable positive samples were investigated by immunohistochemistry (IHC) for *Parachlamydia sp*. using the Dako ChemMate detection Kit (Dako, Heidelberg, Germany) according to the manufacturer's instructions. A mouse polyclonal antibody to detect *Parachlamydia sp.* was applied and IHC was performed as previously described (Ruhl et al., 2009).

The serum samples were tested with two commercially available antibody-detecting ELISA assays, both specific for *C. abortus*: the Pourquier ELISA *Chlamydophila Abortus* (Institute Pourquier) and the ID Screen *Chlamydia abortus* ELISA (ID Vet Innovative Diagnostics).

Details of deer positive for *Chlamydiaceae* by real-time PCR and/or *C. abortus* specific ELISA are shown in Table 1 *(n=7)*. Five out of 717 samples (0.7%) originating from four deer (2.5%) were positive for *Chlamydiaceae* by real-time PCR. Two out of 146 sera were positive (1.4%) by ELISA. Positive samples were found in EDTA blood of roe deer *(n=1)* and in fecal samples of roe deer *(n=2)* and red deer *(n=2)*. The positive EDTA blood and one positive fecal sample originated from the same animal (roe deer no. 1). The five samples

[1]See: http://blast.ncbi.nlm.nih.gov/Blast.cgi

positive for *Chlamydiaceae* by real-time PCR were investigated by AT Microarray, but the chlamydial species identification was not possible as none of the samples hybridized to any of the probes present on the microchip. The sequencing of one fecal sample (red deer no. 3) revealed 100% homology to *C. pecorum*. The 16S rRNA product of EDTA blood *(n=1)* and feces *(n=2)* (roe deer no. 1 and 2) were 100% identical to each other, but did not align completely to any known sequence on BLAST GenBank. The highest homology was 94% to *C. muridarum* followed by 93% to *C. trachomatis* and *C. suis*, respectively. The sequence of one fecal sample (red deer no. 4) could not be analyzed due to poor DNA quality.

Details of cases positive for *Parachlamydia sp*. either by real-time PCR or IHC are shown in Table 2 *(n=6*). By real-time PCR, four samples (eye swabs: *n=3* and lung: *n=1*) originating from four red deer (animal no. 8 to 11) were clearly positive with Ø Ct values ≤ 35.0 corresponding to a copy number above 100. This leads to an occurrence of 2.4% *Parachlamydia sp.* positive deer for the investigated red and roe deer (*n=163*). Additionally, 64 samples (eye swabs: *n=46*; feces: *n=15*; liver: *n=1* and lung: *n=2*) of 48 animals (red deer: *n=25* and roe deer: *n=23*) had Ø Ct values between 35.0 and 38.0 and are considered as questionable positive. From 52/163 (31.9%) positive or questionable positive red and roe deer, 19 deer were concurrently positive or questionable positive at least in two different samples. Of the real-time PCR positive or questionable positive organ samples (liver: *n=1*; lung*: n=3*) of three red deer (no. 11-13), two corresponding formalin-fixed and paraffin-embedded lung samples (red deer no. 12 and 13) were positive by IHC (Figure 1), whereas liver and lung of deer no. 11 were negative.

To our knowledge, this is the first report of *Parachlamydia* in a wild ruminant species. *Parachlamydia* in bovine abortion from the same alpine area in Switzerland has been reported

(Ruhl et al., 2009). Considering only real-time PCR positive deer with Ct values ≤ 35.0 would result in a prevalence of 2.4%. Including the questionable positive deer samples with Ct values between 35.0 and 38.0 would result in a high prevalence of 31.9%. Almost 40% of the positive and/or questionable positive deer had a positive and/or questionable positive result in at least two samples, mostly in eye swabs *(n=49)* or fecal samples *(n=15)*. The question whether *Parachlamydia* is of pathogenic potential in wild ruminants as suggested in domestic ruminants, or if they are only asymptomatic carriers of *Parachlamydia,* remains yet unresolved. Possible fecal excretion of *Parachlamydia* is supported by its detection in fecal samples. We detected *Parachlamydia* positive or questionable positive eye swabs *(n=49)* in red and roe deer but no obvious eye lesions were recorded by the hunters assuming there is no association between clinical ocular disease and the detection of parachlamydial DNA in the eyes of red and roe deer. Similarly, no clear association between *Chlamydia*-like organisms and ocular disease in sheep was reported by Polkinghorne et al. (2009). The real-time PCR positive or questionable positive lung *(n=3)* and liver *(n=1)* in red deer indicate a systemic infection of *Parachlamydia* in a limited number of infected animals. Positive and questionable positive real-time PCR results were confirmed by IHC in lungs of two red deer; the labeling was mainly intracytoplasmic in bronchial epithelial cells, pneumocytes type II and alveolar macrophages. This is consisting with previous in vitro studies showing that *P. acanthamoebae* is able to enter and replicate within human pneumocytes (Casson et al., 2006) and macrophages (Greub et al., 2003).

A very low seroprevalence for *C. abortus* (0.7%) was found in the investigated red and roe deer. This is in concordance to the above-mentioned previous studies in Alpine ibex and chamois, where the same methods revealed occurrences for *Chlamydiaceae* of 1.5% and 1.4%, respectively (Holzwarth et al., 2011a, b). By real-time PCR, four out of 163 (2.5%)

investigated red and roe deer were positive for *Chlamydiaceae*. Fecal samples *(n=4)* had most frequently positive results, followed by EDTA blood *(n=1)*. One fecal sample was clearly *C. pecorum* positive while the other three fecal samples and blood sample were classified within the family of *Chlamydiaceae,* most closely related to *C. muridarum*. The low occurrence for *Chlamydiaceae* (2.5%) is in concordance with previous investigations in the same alpine area in Switzerland, where seven out of 412 Alpine ibex (1.7%) and three out of 79 Alpine chamois (3.8%) were positive for *Chlamydiaceae* (Holzwarth et al., 2011a, b). In contrast to ibex and chamois, *C. abortus* DNA was not found in any deer sample. The finding of *C. pecorum* in feces of a red deer was not a particular unusual finding, as it is known that domestic ruminants can harbor *C. pecorum* in their intestine (Mohamad and Rodolakis, 2010), possibly contaminating pastures and being an infection source for wild ruminants. Feces and blood samples of two roe deer showed exactly the same 16S rRNA sequences most closely related (94%) to *C. muridarum*. These roe deer were sampled in different valleys, on different days by different hunters, implying the occurrence of a possible yet unclassified chlamydial species in roe deer at two different locations in Switzerland. The clinical impact of such non-classified *Chlamydiaceae* species is not yet known.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1

Details of animals *(n=7)* positive or questionable positive by either serology for *C. abortus* and/or real-time PCR for *Chlamydiaceae*

Case no.	Species	Serology		Real-time PCR and chlamydial species identification			
		ELISA ID Screen (% Value)	ELISA Pourquier (% Value)	Real-time PCR (Ø Ct Value)	16S rRNA PCR[a]	Homology (%)	Positive Material
1	Roe deer	neg	neg	pos (32.1/29.3)	*C. muridarum*	94/94[b]	Blood/Feces
2	Roe deer	pos (281)	neg	pos (26.9)	*C. muridarum*	94[b]	Feces
3	Red deer	neg	neg	pos (33.3)	*C. pecorum*	100	Feces
4	Red deer	neg	neg	pos (34.8)	neg	-	Feces
5	Roe deer	pos (155)	neg	neg	nd	-	-
6	Roe deer	quest (56)	neg	neg	nd	-	-
7	Roe deer	quest (55)	neg	neg	nd	-	-

pos: positive; neg: negative; quest: questionable positive; nd: not done

[a] Closest match on BLAST

[b] GenBank accession No. AE002160.2

Table 2

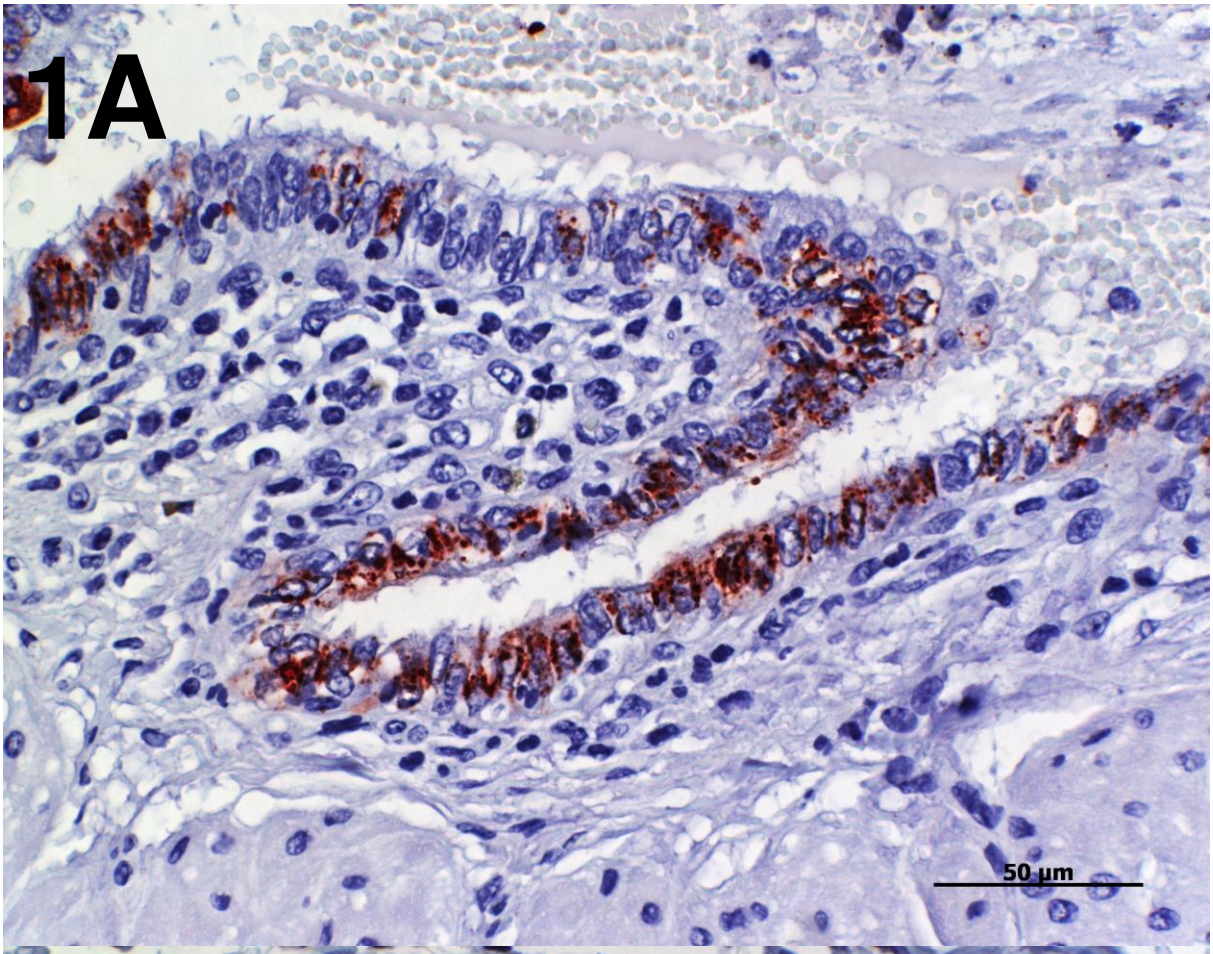
Details of animals *(n=6)* positive by real-time PCR *(n=4)* or immunohistochemistry (IHC) *(n=2)* for *Parachlamydia sp.*

Case no.	Species	Material	Real-time PCR (Ø Ct Value)	IHC
8	Red deer	Eye swab	pos (23.5)	nd
9	Red deer	Eye swab	pos (34.5)	nd
10	Red deer	Eye swab	pos (34.8)	nd
11	Red deer	Lung, liver	pos (34.5)	neg
12	Red deer	Lung	quest (37.5)	pos
13	Red deer	Lung	quest (37.7)	pos

pos: positive; quest: questionable positive; neg: negative; nd: not done

Figure legend

Fig. 1. Formalin-fixed and paraffin-embedded sections of lung tissue from red deer, case no. 13 (A) and no. 12 (B), were investigated by immunohistochemistry for *Parachlamydia sp*. using the Dako ChemMate detection Kit (Dako) according to the manufacturer's instructions. A mouse polyclonal antibody to detect *Parachlamydia sp.* was applied. A. Presence of positive granular reaction intracytoplasmic in bronchiolar epithelium cells. B. Presence of positive granular reaction intracytoplasmic in alveolar macrophages.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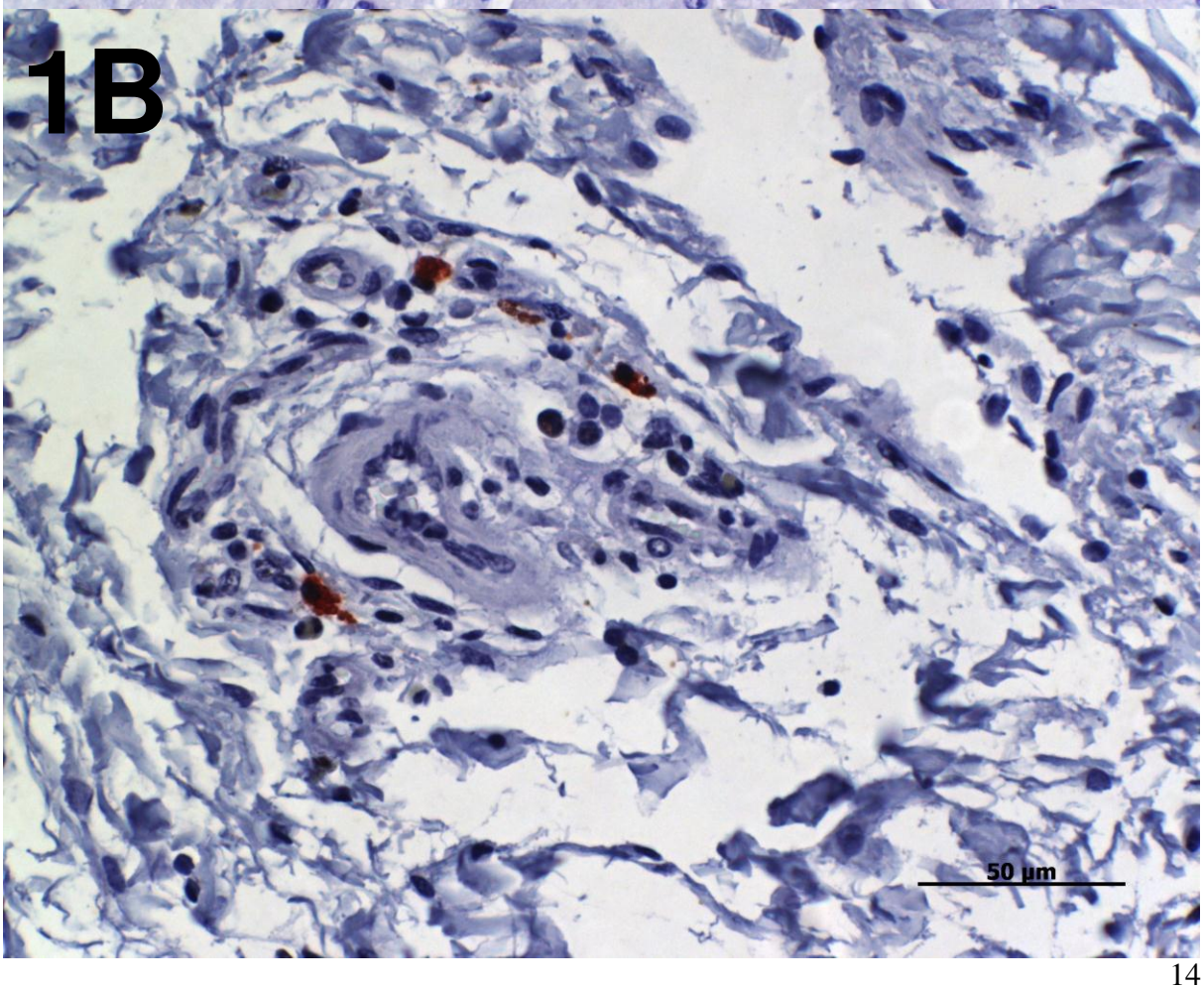
1B
50 µm